C. elegans clk-2, a gene that limits life span, encodes a telomere length regulator similar to yeast telomere binding protein Tel2p

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An important quest in modern biology is to identify genes involved in aging. Model organisms such as the nematode *Caenorhabditis elegans* are particularly useful in this regard. The *C. elegans* genome has been sequenced [1], and single gene mutations that extend adult life span have been identified [2]. Among these longevity-controlling loci are four apparently unrelated genes that belong to the *clk* family [3–5]. In mammals, telomere length and structure can influence cellular, and possibly organismal, aging [6]. Here, we show that *clk-2* encodes a regulator of telomere length in *C. elegans*.

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Results and discussion

Normal somatic cells do not divide indefinitely due to a process termed replicative senescence [7]. Owing to the biochemistry of DNA replication, telomeres, the ends of chromosomes, shorten progressively with each cell division. It has been suggested that telomere shortening disrupts telomere structure and organization, which in turn can influence cellular, and possibly organismal, aging [6]. These findings led us to ask whether there is a connection between telomeres and aging in the soil nematode *Caenorhabditis elegans*.

We examined a mixed-stage population of worms of several long-lived *C. elegans* mutants for changes in telomere length by Southern blotting (Figure 1a; [2]). Among these mutants, animals with the *clk-2(qm37)* mutation, which was identified by a genetic screen for maternal-effect viable mutants [3] and which conferred a longer life span [4], had significantly shorter telomeres relative to wild-type (N2) animals (Figure 1a).

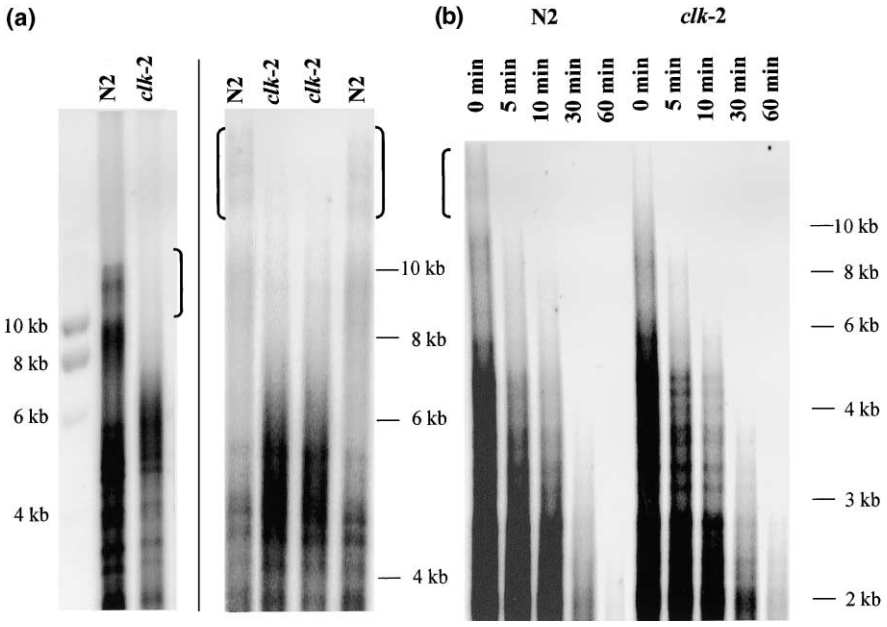
C. elegans telomeres generally appear as a diffuse signal ranging from 4 to 9 kb on low-resolution Southern blots [8, 9]. However, high-resolution Southern blots of HinfI-digested DNA probed with the telomeric repeat $(GCCTAA)_4$ showed that wild-type animals had somewhat longer telomeres (>10 kb; Figure 1a). Notably, in contrast to wild-type telomeres, telomeres from *clk-2(qm37)* mutants were shorter, specifically lacking the high-molecular weight bands (>10 kb). Analyses of multiple independently isolated DNA samples confirmed these results.

To determine whether the high-molecular weight bands were telomeric ends, we assessed their sensitivity to Bal31 exonuclease digestion [10]. The signals at 6 kb and higher were most sensitive to digestion, indicating that they derive from telomeres. Furthermore, the >10 kb signals that were present in N2 DNA but missing in *clk-2(qm37)* DNA were highly sensitive to digestion (Figure 1b). Densitometry on the Bal31-sensitive signals showed that wild-type telomeres ranged from 6 to 12 kb, whereas *clk-2* telomeres ranged from 6 to 9 kb. Weighted averages were difficult to determine owing to the diffuseness of the signals, but were roughly 10 kb and 6–7 kb for wild-type and mutant telomeres, respectively. We conclude that *clk-2* animals have shorter telomeres than wild-type animals.

Based on these results, we reasoned that a mutation in a protein that regulates telomere length might be responsible for the *clk-2* phenotypes. Genetic mapping had placed *clk-2(qm37)* between *osm-10* and *lin-39* [3, 4, 11]. We examined the 51 open reading frames (ORFs) in this interval, and we identified a predicted gene, C07H6.6, that had sequence similarity to Tel2p, a regulator of telomere length in *S. cerevisiae* [12–14].

We identified sequence homologs of this ORF in several species using PSI-BLAST as implemented at the NCBI's www interface to protein-protein BLAST. With default parameter settings, the program was run until convergence such that members of the CKL-2/Tel2p sequence family had E values smaller than 6×10^{-23}. A hidden Markov model (HMM) [15] for this family was trained using the PSI-BLAST pairwise alignment as a starting point. The HMM-generated multiple sequence alignment showed that C07H6.6, Tel2p, and related sequences are similar over nearly 400 amino acids (Figure 2). Est1p, an *S. cerevisiae* single-strand telomeric DNA binding protein [16], did not appear to belong to this family from a statistical perspective, but its alignment to the CLK-2/

Figure 1



clk-2 mutants have shorter telomeres than wild-type animals. **(a)** Genomic DNA was isolated from wild-type N2 and *clk-2*(*qm37*) animals, digested, separated by electrophoresis through agarose gels, and stained with ethidium bromide to confirm integrity. DNA (4 μg) was digested overnight with HinfI and separated on 25 × 20 cm 0.5% agarose-0.5X TBE gels for 23 hr at 1 V/cm. After staining, the gel was dried at 60°C for 1 hr, denatured in 0.5 M NaOH, 1.5 M NaCl for 15 min, neutralized with 0.5 M Tris (pH 7.0), 1.5 M NaCl for 15 min, and rinsed with water. The gel was placed in a hybridization bag with prewarmed hybridization solution (1 mM EDTA, 0.5 M Na_2HPO_4 [pH 7.2], 1% BSA, and 7% SDS) for 2 hr, radiolabeled telomeric probe was added, and the gel was incubated with shaking at 37°C for 12–16 hr. The telomeric probe was a $(GCCTAA)_4$ oligonucleotide, end-labeled with ^{32}P. After hybridization, the gel was washed with 2 l prewarmed washing solution (0.5X SSC, 0.1% SDS) at 37°C. The radioactive signals were visualized with a PhosphorImager and quantified with ImageQuant. The signals generated by N2 DNA but not by *clk-2* DNA are indicated by the brackets. Very similar results were obtained with seven independently isolated pairs of N2 and *clk-2* DNA samples from different generations. Three such sample pairs, run on two different gels (separated by the vertical line; note the different spacing of the size markers), are shown. In addition, the DNA was digested with another 4 base cutter restriction enzyme (Sau3A), with similar results (not shown). **(b)** To determine whether the signals absent from *clk-2* DNA represent telomeres, genomic DNA (15 μg) was digested with 10 U Bal31 nuclease (GIBCO-BRL) for varying intervals up to 1 hr. Aliquots containing 3 μg were removed after 0, 5, 10, 30, and 60 min, and digestion was stopped by adding EGTA (pH 8.0) to a final concentration of 20 mM. After extraction with phenol/chloroform/isoamyl alcohol, aliquots were ethanol-precipitated. DNA pellets were resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and digested with HinfI overnight. After electrophoresis, in-gel hybridization was performed as described above.

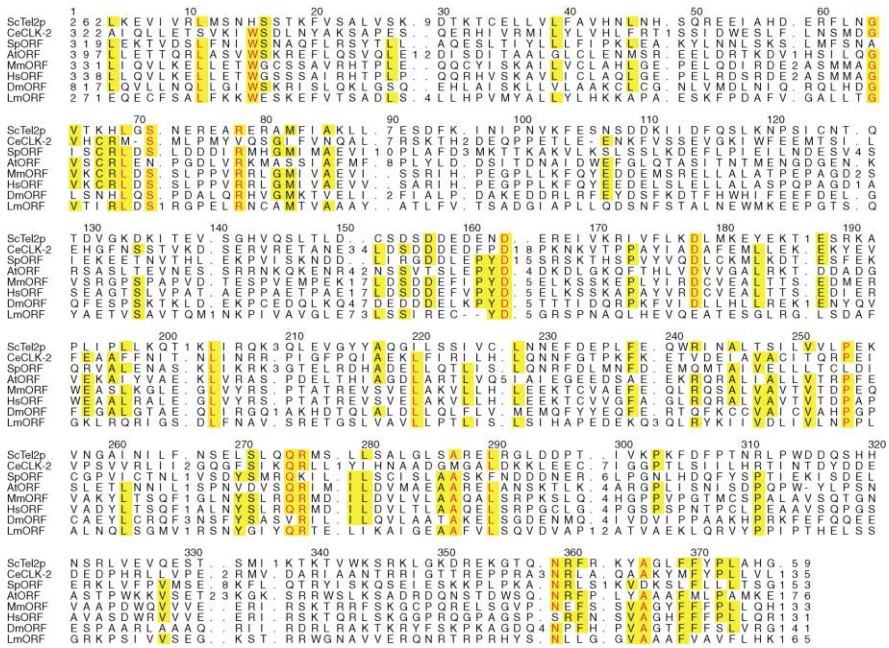
Tel2p family suggested a possible relationship (data not shown).

Given that the Tel2p-1 mutation can shorten telomeres in yeast, we tested the possibility that the candidate *TEL2* homolog, C07H6.6, is the *clk-2* gene. We first asked whether the embryonic lethality at 25°C of *clk-2(qm37)* animals is rescued by DNA transformation [17] with a wild-type C07H6.6 gene. C07H6.6 likely resides in an operon, downstream from another ORF, C07H6.8. To express both genes from their endogenous promoter, the genomic region spanning both ORFs was amplified by PCR (Figure 3). The full-length PCR product was injected into *clk-2(qm37)* animals. It completely rescued the embryonic lethality at 25°C. By contrast, a truncated PCR product lacking most of the C07H6.6 gene failed to rescue *clk-2* mutants (Figure 3). These results suggest that the ORF C07H6.6 is the *clk-2* gene.

To identify the mutation in C07H6.6 potentially responsible for the *clk-2* phenotypes, we sequenced the C07H6.6 genomic region from *clk-2*(*qm37*) mutants. We found a single point mutation (G to A), which substitutes tyrosine for cysteine at amino acid (aa) 772 in the predicted CLK-2 protein sequence. We also sequenced a cDNA (yk447b4) (from Y. Kohara, National Institute of Genetics, Japan) corresponding to the C07H6.6 ORF (GenBank accession number AJ320257). The cDNA and genomic sequences agreed, aside from the first four bases in the cDNA, which likely correspond to the SL2 *trans*-splice leader. The size of the cDNA (2.8 kb) corresponded to that of the mRNA detected on Northern blots (data not shown), suggesting that yk447b4 is a full-length cDNA clone. The cDNA sequence predicts an 877 aa protein, slightly longer than the 837 predicted by GeneFinder [11]. Our sequence identified 40 aa (residues 258–277 and 342–361) that are missing in the GeneFinder-predicted sequence and 9 mismatches (between aa 329 and 341) between the sequences predicted by the cDNA and GeneFinder [11].

The conservative nature of the missense mutation in *clk-2(qm37)* and the temperature-sensitive phenotype of *clk-2(qm37)* animals suggest that this allele might not cause a complete loss of protein function. We obtained evidence for this idea by using RNA-mediated interference (RNAi) to deplete the C07H6.6 gene product [18]. We injected

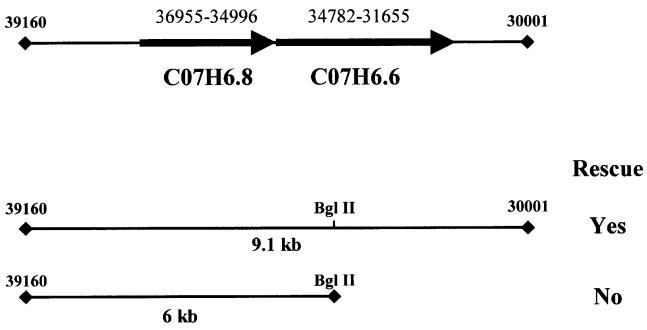
Figure 2



An HMM-generated multiple sequence alignment of the CLK-2/Tel2p sequence family. Highly conserved (red letters) and well-conserved (black letters) alignment positions are shown shaded in yellow. Numbers within the alignment denote the number of residues not shown explicitly and those which align to the insert state of the HMM. The sequences shown are ScTel2p, *Saccharomyces cerevisiae* Tel2p (Swiss-Prot code TEL2_YEAST); CeCLK-2, *Caenorhabditis elegans* CLK-2 (GenBank accession number AJ320257); SpORF, *Schizosaccharomyces pombe* ORF SPAC458.03 (GenBank accession number CAB93845); AtORF, *Arabidopsis thaliana* ORF T29H11_10 (GenBank accession number CAB88328); MmORF, *Mus musculus* ORF AK004582 (GenBank accession number BAB23388); HsORF, *Homo sapiens* ORF KIAA0683 (GenBank accession number BAA31658); DmORF, *Drosophila melanogaster* ORF CG13854 (GenBank accession number AAF55990); and LmORF, *Leishmania major* ORF L7480.5 (GenBank accession number AC078900_5). The Cys to Tyr substitution caused by the *clk-2*(*qm37*) mutation is located in the 135 amino acid region at the C terminus that is not shown. The amino acids between positions 329 and 341 are those derived from the cDNA sequence.

double-stranded RNA corresponding to the C07H6.6 cDNA into wild-type animals and examined their progeny at 20°C. Embryos laid within 48 hr after injection, during which time RNAi is not fully functional, showed retarded development (Table 1). Moreover, the injected animals showed a reduced defecation rate and reduced fertility (Table 1). These phenotypes closely resemble those reported for *clk* mutants [3–5, 19]. All the embryos that were laid >48 hr after injection died (data not shown). These findings suggest that the *clk-2*(*qm37*) mutation causes a partial loss of *clk-2* function, with complete loss of function being embryonic lethal.

Figure 3



The ORF C07H6.6 is the *clk-2* gene. DNA transformation rescue. A wild-type genomic fragment spanning both C07H6.8 and C07H6.8 was amplified by PCR and injected into *clk-2* animals. Similarly, a truncated genomic fragment removing most of the ORF C07H6.6 was injected into *clk-2* animals. Three transgenic lines were established per DNA construct and tested for the ability to rescue embryonic lethality at 25°C. "Yes" denotes rescuing activity; "No" denotes no rescue. Injection of buffer did not rescue the phenotype (not shown). The upper image depicts the genomic region encompassing the putative *clk-2* locus, and the lower images depict the PCR products injected into the *clk-2* animals.

Consistent with this hypothesis, when we injected double-stranded C07H6.6 RNA into *clk-2*(*qm37*) mutant animals, all the embryos laid within 12 hr after injection died (data not shown). Moreover, when we injected the RNA into wild-type animals at 25°C, 50% of the embryos laid within 24 hr after injection died, and those that reached adulthood were sterile, thin, and pale (data not shown), similar to the *clk-2*(*qm37*) phenotype at 25°C [3]. These phenotypes are similar to those recently uncovered by a large-scale analysis of inactivation of ORFs by RNAi [20]. In contrast to animals injected with C07H6.6 RNAi, animals injected with RNAi for C07H6.8 (the upstream gene in the same operon) showed normal development, similar to that of wild-type animals [20]. In addition, inactivation of telomerase (CeTERT) by RNAi had no discernible effect for several generations [20], suggesting that the phenotypes of animals injected with C07H6.6 RNAi were

Table 1

RNA-mediated genetic interference (RNAi).

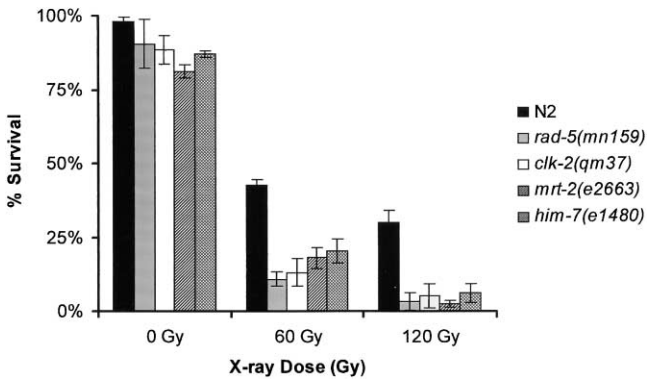
	RNAi <48 hr	Embyogenesis[a] (hr)	Defecation[b] (sec/cycle)	Fertility[c] (number of eggs)
N2 (n = 10)	+	23.2 ± 2.3	98.0 ± 20.3	43.0 ± 10.2
N2 (n = 10)	−	13.1 ± 1.1	51.8 ± 4.3	313.4 ± 30.5

[a]Eggs (100 from control and 100 from RNAi-injected animals) that were laid within 48 hr after injection were examined for survival and time to hatching (hr). [b]Five control and five RNAi-injected animals were examined for defecation rate. Shown is the time per defecation cycle (sec/cycle), averaged over three cycles per animal. [c]Eggs from 10 control and 10 RNAi-injected animals were collected and assessed for fertility by counting the number of eggs/animals over 96 hr after injection. Results for the control and RNAi-injected animals were statistically significant ($p \leq 0.00003$ for embryogenesis, $p \leq 0.0005$ for defecation rate, and $p \leq 0.00003$ for fertility). The cDNA clone (yk447b4) containing the C07H6.6 ORF was used as a template for RNA synthesis. Single-stranded RNAs were synthesized using the RiboMAX RNA production system (Promega, Madison, Wisconsin, USA). Double-stranded RNAs were produced by annealing the complementary single-stranded RNAs by heating to 92°C and gradually cooling. Double-stranded RNAs were diluted (1 μg/μl) into injection buffer [17] and injected into the intestines or gonads of young adult N2 hermaphrodites. Injected animals were allowed to recover at 20°C and analyzed. Ten N2 animals were injected with double-stranded C07H6.6 RNA (+), and 10 control animals were injected with buffer alone (−).

not due to nonspecific consequences of inactivating a gene involved in telomere metabolism. Taken together, these findings provide additional support that the C07H6.6 ORF is the *clk-2* gene.

Recent findings suggest that short telomeres can confer sensitivity to ionizing radiation (IR). Thus, sixth generation telomerase-deficient mice, which have substantially shorter telomeres than the second generation or wild-type mice, were much more sensitive to IR-induced killing [21]. We therefore asked whether *clk-2* mutant animals were hypersensitive to IR. We irradiated wild-type and *clk-2* L4 larvae with X-rays (60 and 120 Gy) (Figure 4). Consistent with their shorter telomeres, *clk-2* animals were hypersensitive to IR, similar to that of three known IR-sensitive mutants (*rad-5*, *mrt-2*, and *him-7*) [22]. Although we cannot exclude the possibility that the radiosensitivity is independent of telomere length, this result is consistent with *clk-2* animals having shorter telomeres.

Figure 4



clk-2 animals are hypersensitive to ionizing radiation. L4 stage N2, *clk-2(qm37)*, *rad-5(mn159)*, *mrt-2(e2663)*, and *him-7(e1480)* animals were X-irradiated at the indicated doses. Eggs were collected between 24 and 36 hr after irradiation and examined for viability. Ten N2, *rad-5 (mn159)*, *mrt-2(e2663)*, and *him-7(e1480)* animals and 20 *clk-2(qm37)* animals were scored at each dose.

Our results suggest that *clk-2* encodes a protein related to the yeast telomere binding protein Tel2p and as such, is the first metazoan Tel2p homolog to be identified. Our results also suggest that the *clk-2*(*qm37*) mutation causes partial loss of function. This mutation is known to retard embryonic and postembryonic development. In adults, this mutation slows physiological rhythmic functions such as defecation, swimming, and pharyngeal pumping and extends adult life span [3, 4, 23]. We show that this mutation also reduces telomere length in adult animals.

Telomere length has been inversely correlated with age in humans and several other species, and short telomeres are proposed to contribute to certain age-related pathologies [6, 7]. However, the relationship among telomere length, the rate of aging, and longevity does not hold for all species [7]. Mice and humans, for example, share many aging phenotypes, yet mice age more rapidly and are shorter-lived despite longer telomeres. Our results indicate that *C. elegans* is another species in which telomere length does not correlate with longevity. How, then, might the telomere phenotype of *clk-2* animals relate to their increased life span? In mammals, short telomeres induce cellular senescence, and the accumulation of dysfunctional senescent cells is thought to contribute to aging [26]. Because DNA damage and other stresses also cause cellular senescence, senescent cells can accumulate in organisms with long telomeres. Thus, short telomeres may contribute to aging indirectly by triggering a cellular response to damage and stress. It was recently proposed that cells sense and respond to telomere structure, rather than to length [6]. According to this idea, telomeres can adopt a capped functional structure or open dysfunctional structure, the latter being favored by short telomeres. We

speculate that the mutant CLK2 protein can stabilize telomeres despite reduced length and can thus stabilize cellular phenotype. Alternatively, the mutant protein may alter chromatin structures that are sensitive to telomere states. Finally, the mutant protein may fail to maintain germline integrity and thus extinguish gonadal and/or sensory neuron signals that shorten life span [24, 25]. However, life span extension caused by germ cell or sensory neuron oblation requires DAF-16 [25] but the longevity conferred by *clk* genes, including *clk-2*, does not [23]. Until the biochemical functions of CLK-2 are elucidated, how it couples telomere metabolism to life span remains speculative.

Acknowledgements

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